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Award Number: DAMD17-99-1-9029

TITLE: Chemokine Receptors and Integrin Function in Prostate Cancer

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REPORT DATE: February 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2000	Annual (15 Jan 99 - 14 Jan 00)

4. TITLE AND SUBTITLE
Chemokine Receptors and Integrin Function in Prostate Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9029

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT: Preliminary data demonstrated that the addition of specific α-chemokines, IL-8 and Gro-α, to prostate carcinoma cell cultures, leads to an increase in the motility and invasion of these cells in vitro. Based on these results, as well as others, we hypothesize that a.) addition of α -chemokines to prostate carcinoma cells stimulates intracellular signaling pathways that lead to enhanced integrin mediated adhesion to ECM components b.) stimulation of chemokine receptors results in the activation of specific rho family of GTPases causing changes in integrin function and c.) inhibition of chemokine-induced activation of specific rho family GTPases will inhibit chemokine induced enhancement of integrin function and tumor cell motility and invasion. These hypotheses will be addressed in two specific aims. In the first specific aim, prostate carcinoma cell lines will be evaluated for the ability to respond to chemokines by measuring adhesion, motility and invasion; response to chemokines will be correlated to expression of specific chemokine receptors by tumor cells. The effect of chemokines on ligand binding by prostate cancer cell integrins will also be measured to determine if chemokines stimulate integrin avidity or affinity. Secondly, gene transfer experiments will be used to determine if specific rho family dominant-negative GTPases inhibit the α-chemokine stimulated increase in integrin function, migration and invasion.

14. SUBJECT TERMS
Prostate

15. NUMBER OF PAGES
19

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

JM N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

JM N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

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PI - Signature 4/30/00 Date

Table of Contents

Introduction

The purpose of the proposed studies is to further understand the molecular mechanisms that govern prostate carcinoma invasion and metastasis. These studies are based on preliminary data in which we had demonstrated that addition of specific soluble factors termed α-chemokines could stimulate the motility and invasion of metastatic prostate cancer cell lines in vitro. These soluble mediators have been implicated in the invasion and metastasis of different tumor types, and they have been implicated in promoting inflammation and/or tumor neovascularization. Our evidence provided in the initial application also implicated these factors as autocrine mediators of tumor behavior. In the first set of studies, we had proposed to further define the mechanism of action of α-chemokines on prostate tumor invasion. We have since determined that α-chemokine treatment of prostate tumors can modulate integrin-mediated adhesion to the glycoprotein laminin. Modulation of integrin function was biphasic, in the sense that α-chemokine treatment stimulated an initial increase in laminin-mediated adhesion, which then subsided back to baseline. These changes occur in the absence of a change in integrin expression levels. The data suggest that α-chemokines can stimulate motility in part by modulating integrin adhesive function, presumably by stimulating signal transduction pathways that lead to changes in integrin avidity.

We also used RT-PCR to determine the nature of the chemokine receptors that are expressed on metastatic PC3-M human prostate carcinomas. The results demonstrated that both CXCR-1 and CXCR-2 receptors are expressed by these cells, indicating that both receptors may be important in mediating prostate tumor motility and invasion. These results were published in the journal The Prostate in October of 1999 (1). One complicating factor in this model system is that the PC3-M cells express high levels of endogenous chemokine. Although this suggests that IL-8 related chemokines may serve as autocrine factors in prostate tumor invasion, it does create certain technical challenges associated with chronic activation of this receptor/ligand pathway in vitro. A copy of the article describing these results is appended to the annual report.

During the second year of funding, we will a.) use antisense approaches to inhibit chemokine receptor expression b.) initiate studies to define which rho-family GTPases are important for α-chemokine mediated tumor cell motility and invasion.

Body

STATEMENT OF WORK

Specific Aim #1: To evaluate the ability of the α- chemokines IL-8 and Gro-α to stimulate integrin mediated adhesion, migration and invasion of human prostate carcinoma cell lines with different invasive or metastatic potential.

Months 1-4: Complete analysis of integrin expression, α- chemokine receptor expression on prostate cancer cell line

Months 2-9: Complete analysis of effects of chemokines on prostate carcinoma cell adhesion, spreading, migration and invasion

Months 4-12: Complete analysis of chemokine effect on integrin mediated affinity and avidity

Month 12: Prepare annual progress report

The studies in Specific Aim #1 of the Statement of Work have been completed and published. The manuscript has been appended that describes these findings. Major conclusions/findings of the manuscript are included in the "conclusions" section, below.

Specific Aim #2: To determine if the α-chemokine induced increase in integrin mediated adhesion is brought about by the action of the rho family of GTPases, we will use a gene transfer approach. This will be done by transfecting dominant negative constructs of rho, rac or cdc-42 GTPases and measuring the effects of α-chemokines on integrin function in invasive and metastatic prostate carcinoma cells

Months 13-24: Transfection of selected prostate carcinoma cell lines with dominant negative constructs of rho, rac and cdc-42 expressing dominant negative or constitutively active versions of these proteins.

Months 13-16: Complete characterization of transfectants for integrin and chemokine receptor expression.

Months 22-30: Analyze dominant transfectants for inhibition of α-chemokine enhanced integrin mediated adhesion, ligand binding, motility and invasion

Month 24: Prepare second annual report and submit competitive renewal application

The studies in the second specific aim are currently underway. Because of the endogenous expression of high levels of IL-8 by these cells, we are first going to use an anti-sense approach to inhibit endogenous receptor expression to determine if this will cause a corresponding decrease in the baseline invasion of these cells. This is supported in part on our observations that CXC antibodies inhibit baseline invasion of these cells (described in accompanying manuscript). We are then prepared to start to evaluate the important rho-family GTPases that are important for IL-8 stimulated invasion. We will start by using constructs that encode dominant negative rho family GTPases (starting with cdc42 and rac-1). We will also directly measure the active state of cdc42 and rac-1, by using a PAK pulldown assay that we have recently adopted (2). Recent work has also implicated these GTPases in gene expression, and our focus on tumor invasion may extend during this year to determining if IL-8 or related chemokines stimulate the expression or activation of specific tumor-associated proteases. We feel this may be more informative than the original plan to solely measure changes in integrin adhesive function as a function of chemokine mediated GTPase activation.

Key Research Accomplishments

a. Demonstration that α-chemokines can stimulate prostate tumor motility and invasion in vitro
b. Identification of the chemokine receptors CXC R1 and CXC R2 expressed by prostate cancer cells
c. Treatment with α-chemokines can stimulate integrin mediated adhesion in the absence of a change in integrin surface expression, suggesting that chemokines change integrin avidity and prostate tumor motility
d. Anti-chemokine receptor antibodies inhibit stimulated and basal tumor cell motility. This suggests that endogenous chemokine expression by prostate tumors can act as autocrine motility/invasion factors. Interfering with receptor may represent a novel therapy for tumor invasion

Reportable Outcomes

a. Manuscript published on above findings. Included in Appendix

Reiland, J, Furcht, L.T. and J.B. McCarthy. 1999. CXC-chemokines stimulate invasion and chemotaxis in prostate carcinoma cells through the CXCR2 receptor. The Prostate. 41:78-88.

Conclusions

The major conclusion of these findings is that chemokines can stimulate prostate tumor motility and invasion. Autocrine expression of chemokines by tumors could serve as autocrine factors for tumor invasion. Furthermore, since chemokines are produced by inflammatory cells, it is also possible that tumor associated inflammation could act to facilitate tumor invasion by increasing the local concentration of inflammatory chemokines. The two major α-chemokine receptors, CXCR1 and CXCR2 were identified on these cells, and anti-CXCR2 antibodies inhibited motility associated with chemokine stimulation. These antibodies also inhibited baseline invasion, suggesting interfering with these receptors could inhibit autocrine and paracrine effects of chemokines on tumor invasion. Work in the upcoming year will focus on further defining the mechanism(s) of action of chemokines, from the standpoint of receptor function and signal transduction mechanisms.

References

1. Reiland, J, Furcht, L.T. and J.B. McCarthy. 1999. CXC-chemokines stimulate invasion and chemotaxis in prostate carcinoma cells through the CXCR2 receptor. The Prostate. 41:78-88.
2. Eisenmann KM, **McCarthy JB**, Simpson MA, Keely PJ, Guan JL, Tachibana K, Lim L, Manser E., **Furcht LT, Iida J**. Melanoma Chondroitin sulfate proteoglycan regulates cell spreading through cdc42, Ack-1 and p130cas. Nat. Cell Biol. 1:507-513, 1999.

Appendix

One publication:

Reiland, J, Furcht, L.T. and J.B. McCarthy. 1999. CXC-chemokines stimulate invasion and chemotaxis in prostate carcinoma cells through the CXCR2 receptor. The Prostate. 41:78-88.

Final Reports

N/A (This is an annual report)

The Prostate 41:78–88 (1999)

CXC-Chemokines Stimulate Invasion and Chemotaxis in Prostate Carcinoma Cells Through the CXCR2 Receptor

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BACKGROUND. Metastasis of prostate carcinoma requires invasion through the basement membrane, a thin extracellular matrix that underlies the epithelial cells, which must be breached by tumor cells invading into surrounding tissue. The CXC-chemokines, which have been shown to promote the migration of neutrophils and carcinoma cells, are candidates to influence prostate carcinoma-cell invasion.

METHODS. CXC-chemokines were examined for the ability to stimulate prostate cell line PC3 invasion in vitro through a reconstituted basement membrane and long-term migration and short-term adhesion to laminin, a major component of the basement membrane.

RESULTS. PC3 cells responded to IL-8 and GROα with a 1.6–2-fold increase in invasion through reconstituted basement membrane. A corresponding 2–3-fold increase in chemotaxis toward IL-8 and GROa was seen on laminin. Anti-CXCR2 antibody inhibited IL-8-stimulated migration. Expression levels of the β_1 integrins were not changed by IL-8, and $\alpha_{6\beta1}$ integrin was used for both stimulated and baseline migration. In addition to the increases in migration and invasion, 2–6-fold transient increases in adhesion on laminin were seen with both IL-8 and GROα.

CONCLUSIONS. These results suggest that the CXC-chemokines stimulate migration and invasion in part by altering the activation state of the β_1 integrins. The CXC-chemokines act on prostate carcinoma cells through the CXCR2 receptor to promote behavior important for metastasis, and as such may be important in prostate carcinoma progression and metastasis. *Prostate 41:78–88, 1999.*

KEY WORDS: **interleukin 8; integrins; laminin; cell adhesion; basement membrane**

INTRODUCTION

IL-8 and GROα are members of a family of CXC-chemokines (CTAP-III, β-TG, NAP-2, PF-4 GROβ, GROγ, IP-10, GCP-2, and ENA-78) defined structurally by four conserved cysteines and functionally by their ability to stimulate chemotaxis of various cells [1,2]. IL-8 and GROα are produced in sites of inflammation by monocytes, macrophages, and nonleukocyte cells such as epithelial and endothelial cells, fibroblasts, and keratinocytes [1]. IL-8 and GROα are

Abbreviations: FACS, fluorescence-activated cell scan; FN, fibronectin; LMN, laminin; mAb, monoclonal antibody; pAb, polyclonal antibody; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reactions; SFM, serum-free medium.

Grant sponsor: National Cancer Institute/National Institutes of Health; Grant number: CA29995; Grant sponsor: DOD; Grant number: PC970519.

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Received 27 July 1998; Accepted 19 April 1999

also produced by tumor cells such as prostate, lung, and melanoma [3–6]. IL-8- and GROα-stimulated adhesion and chemotaxis are important for neutrophil activation and infiltration [7]. In addition, IL-8 and GROα stimulate the migration of other cell types such as T lymphocytes, smooth muscle cells, keratinocytes, and endothelial and tumor cells including melanoma and breast carcinoma [8–11].

IL-8 and GROα stimulation of adhesion and migration may be an important factor in tumor progression. CXC-chemokine expression is correlated with tumor progression in melanomas, which exhibit increased expression of IL-8 when compared to normal melanocytes [5]. Overexpression of GROα in melanocytes results in increased tumorigenicity [12]. Increased expression of IL-8 message and protein in tumors also correlates with increased experimental metastatic potential of melanoma cells in nude mice [6]. IL-8 is also implicated in metastasis of lung carcinoma cells, as neutralizing IL-8 antibodies or IL-8 receptor antisense oligonucleotide inhibit growth and metastasis of lung carcinoma cells in vivo [3,13]. These studies suggest that the CXC-chemokines play a critical role in tumor progression.

IL-8 and GROα act through a family of G-protein-coupled receptors designated the "CXCR receptors." CXCR1 (IL-8RA) binds IL-8 with high affinity but binds other members of the CXC-chemokine family with low affinity. In contrast, CXCR2 (IL-8RB) binds IL-8, GROα, NAP-2, ENA 78, and GCP-2 with high affinity [14,15]. Both receptors mediate IL-8-stimulated chemotaxis in CXCR1 or CXCR2 transfected Jurkat cells [16], and neutrophils can use both receptors to promote chemotaxis [17]. Chemokine binding to CXCR1 and CXCR2 stimulates several signal transduction pathways, including release of intercellular stores of calcium and activation of PLC [18–20], ERK [21], Rho [22], and phosphoinositide metabolism [23]. IL-8 binding to either receptor stimulates changes in adhesion receptor expression, including upregulation of CD11b/CD18, and induction of L-selectin shedding from the plasma membrane on neutrophils [24–26]. It also stimulates cytoskeletal rearrangement, pseudopodia formation, and morphological polarization [1].

Since adhesion and migration are important components of invasion into local or distant sites during prostate tumor metastasis, we examined the ability of the CXC-chemokines to stimulate prostate carcinoma adhesion, migration, and invasion. Invasion was examined using a reconstituted basement membrane, while adhesion and migration were examined using substrata coated with laminin, a major component of the basement membrane. We have found that prostate carcinoma cells are able to respond to two members of the CXC-chemokine family, IL-8 and GROα. Both chemokines stimulate adhesion and migration of prostate carcinoma cells on laminin and also increased tumor cell invasion through reconstituted basement membrane. The effects of IL-8 and GROα are mediated through the CXCR2 receptor, as PC3 cells express CXCR2 mRNA, and neutralizing antibodies for CXCR2 inhibit IL-8-stimulated adhesion and migration. The results suggest that CXCR expression in prostate carcinoma may facilitate local invasion and metastasis.

MATERIALS AND METHODS

Cell Culture

Prostate adenocarcinoma cell line PC3 was obtained from the ATCC (Rockville, MD) and maintained in F12 Kaighn's modification with 7% fetal bovine serum. Cells were plated 1–2 days prior to experiments and used at approximately 50% confluence.

Reagents

Mouse EHS laminin [27] and fibronectin [28] were prepared as described. IL-8 and GROα were purchased from R&D Systems (Minneapolis, MN) or Peprotech (Rocky Hill, NJ). Primers for PCR were produced by the University of Minnesota Microchemical Facility (Minneapolis, MN). Commercially available monoclonal antibodies to IL-8 and CXCR2 (R&D Systems) and alpha 6 integrin (Immunotech, Inc., Westbrook, ME) were used. Purified antibody against the beta 1 integrin subunit (P5D2) was produced and characterized as previously described [29].

Detection of IL-8 Receptor RNA

Total RNA was isolated from PC3 cells by guanidinium-phenol-chloroform extraction, using the Totally RNA Kit according to the manufacturer's instructions (Ambion, Austin, TX). First-strand cDNA was synthesized from total RNA using AMV reverse transcriptase (Gibco BRL, Grand Island, NY) according to manufacturer's instructions. Specific cDNAs for CXCR2 were amplified by PCR with primers 5'CCTTTTCTACTAGATGCCGC and 5'GCGGCATCTAGTAGAAAAGG [18]. Specific cDNAs for CXCR1 were amplified by PCR with 5'GAAGAAGAGCCAACAAAG and 5'CTTTGTTGGCTCTTCTTC. Amplification with PCR Master Mix (Gibco BRL) was performed with 30 cycles at 94°C, 58°C, and 70°C (1 min each) for CXCR2, and 94°C, 60°C, and 70°C (1 min each) for CXCR1, followed by 12 min of 72°C in a Perkin Elmer Cetus (Foster City, CA) DNA Thermocycler 480. Am-

plified products were analyzed by agarose gel electrophoresis.

Cell Adhesion Assay

Microtiter plates (Immobilon, Chantilly, VA) were prepared for adhesion by incubating wells with 100 μl laminin in PBS for 18–24 hr at 37°C in a humidified incubator. Solutions were removed and excess sites were blocked with 1 mg/ml bovine serum albumin (BSA) in PBS for 1–2 hr at 37°C [27]. Subconfluent PC3 cells were released with brief treatment of 0.25% trypsin-EDTA. The trypsin was inhibited with 0.5 mg/ml soybean trypsin inhibitor in SFM (F12 Kaighn's modification with 0.1% BSA, 1 mM sodium pyruvate, and 50 μg/ml gentamicin), and the cells were washed with SFM and resuspended in adhesion medium (SFM with 4 mM HEPES). Cells were incubated with the indicated concentration of chemokine for 0–30 min at 37°C in 5% CO_2. Neutralizing antibodies were added prior to the chemokine when appropriate. Calcein-AM (Molecular Probes, Eugene, OR), a fluorescent probe which is internalized and hydrolyzed by intracellular esterases producing a fluorophor which emits at 530 nM, was added to a final concentration of 1 μg/ml. Approximately 1,000 cells/well were then added to adhesion plate and incubated 37°C for 35 min, gently washed 2–3 times, and quantified in a Cytofluor II fluorescent plate-reader (Biosearch, Inc., Bedford, MA). Separate standard curves were performed for each IL-8 treatment; however, no effect was seen on the fluorescent intensity of the dye with the different chemokine treatments.

Cell Migration Assay

Cell motility was assayed in 48-well microchambers (Neuroprobe, Bethesda, MD) utilizing 8-μm pore size polyvinyl pyrrolidone-free polycarbonate filters (Nucleopore, Pleasanton, CA) precoated on both sides with the indicated concentrations of laminin in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) for 16–20 hr. The lower wells of the microchamber were filled with SFM and the indicated concentration of chemokine. Adherent PC3 cells were released as for the adhesion assay and resuspended at 4×10^5 cells/ml in SFM, and 50 μl of cells were added to the upper compartment. Neutralizing antibodies were added to the cells before addition to the chamber when appropriate. Cells were allowed to migrate for 6 hr at 37°C in a 5% CO_2 incubator, and cells that had migrated to the underside of the filter were stained and counted.

Cell Invasion Assay

Cell invasion chambers were prepared according to the manufacturer's instructions (Collaborative Biomedical Products, Bedford, MA). PC3 cells (200,000 cells/well) in SFM were added to the upper chamber. SFM containing the indicated concentrations on chemokine and 25 μg/ml fibronectin was added to the lower chamber. Invasion to the lower chamber was assayed after 20–30 hr by visual quantification of cells adhering to the underside of the filter. Experiments were performed three times, and representative experiments are shown.

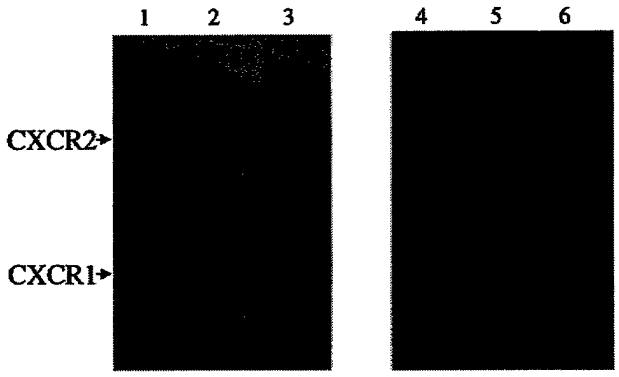

Fig. 1. PC3 cells express CXCR2 mRNA. Total RNA was isolated from PC3 cells (**lanes 1, 2, 4, 5**) or neutrophils (**lanes 3, 6**). cDNA was made using reverse transcriptase and used for RT-PCR with primers for CXCR2 (lanes 1, 3) or CXCR2 (lanes 4, 6). The no-RT control omitted the reverse transcriptase when making the cDNA (lanes 2, 5).

RESULTS

PC3 Cells Express CXCR2 and CXCR1 mRNA

To determine if PC3 cells could express message for receptors that bind the CXC-chemokines, mRNA was isolated from PC3 cells and RT-PCR was performed for CXCR1 and CXCR2 receptors, using primers from unique areas in the receptor sequences. Primers were chosen so that the resulting amplified DNA would be 967 bp for CXCR2 and 518 bp for CXCR1. CXCR1 and CXCR2 RNA transcripts were detected in the PC3 cells (Fig. 1). Neutrophils were used as a positive control and, as expected, expressed mRNA for both receptors. Control reactions without reverse transcriptase did not have amplified message.

CXC-Chemokines, IL-8, and GROα Stimulate Invasion Through a Reconstituted Basement Membrane

The basement membrane underlies the endothelium in the prostate, presenting a barrier that the prostate carcinoma cell must cross in order to metastasize. Invasion through a reconstituted basement membrane is one of the best in vitro indicators of tumor progres-

sion [30]. Since PC3 cells express CXCR2, we tested invasion with or without IL-8 and GROα, two ligands for CXCR2 shown to be chemotactic agents in other cell types. PC3 cells were added to the upper chamber of the invasion chambers, and IL-8 or GROα were added as chemoattractants to the lower part of the chamber. After 24 hr, cells that invaded through the reconstituted basement membrane were visually quantified. IL-8 and GROα were both effective in increasing PC3 invasion, stimulating invasion 1.8-fold ($P < 0.03$) and 1.7-fold ($P < 0.05$) over untreated controls, respectively (Fig. 2). GROα and IL-8 did not stimulate growth, as measured by accessing cell number in the PC3 cells under conditions similar to those in the invasion assay, indicating that chemokine-stimulated invasion is not due to increased cell proliferation (data not shown). As found in neutrophils, IL-8-induced migration is chemotactic in nature, since IL-8-induced migration was higher if an IL-8 gradient was established rather than if IL-8 was added together with the cells to stimulate random migration (data not shown). IL-8 has been shown to increase the expression of adhesion receptors in neutrophils; therefore, we examined whether IL-8 or GROα stimulated changes in the integrins which bind laminin and type IV collagen, major components of the basement membrane. PC3 cells stimulated with GROα or IL-8 did not change their expression of β_1, β_4, α_2, α_3, or α_6 integrins as measured by flow cytometry (data not shown).

IL-8 and GROα Stimulate Migration on Laminin, a Major Component of the Basement Membrane

CXC-chemokines have been shown to stimulate migration in a variety of cell types. In order to determine if chemotactic properties could contribute to the IL-8 stimulation of invasion, we assessed the ability of IL-8 to stimulate PC3 migration. Laminin, a major component of the basement membrane, was coated on both sides of the migration filter. This provided a relevant matrix to support cellular migration. However, since it was coated on both sides of the filter, it did not act as a haptotactic reagent. Also, suboptimal concentrations of laminin were used to minimize the potential contribution of spontaneous random migration on laminin. At these concentrations, LMN was able to support low levels of random migration. IL-8 was added to the lower compartment of a modified Boyden chamber to act as a chemotactic agent, and PC3 cells were added to the upper compartment and allowed to migrate for 6 hr. Over a range of 0.2–20 nM, IL-8 was chemotactic for PC3 cells migrating on laminin ($P < 0.05$ at 0.2 nM and $P < 0.001$ at 2 and 20 nM) (Fig. 3). IL-8 concentrations used for stimulating PC3 cell migration were similar to concentrations of IL-8 used to stimulate neutrophil and T-cell transmigration through endothelial cells [9,10,31]. IL-8-stimulated migration was inhibited by anti-IL-8 mAbs, demonstrating the specificity of the reaction (data not shown). IL-8 therefore acts as a chemoattractant for PC3 prostate carcinoma cells under these conditions.

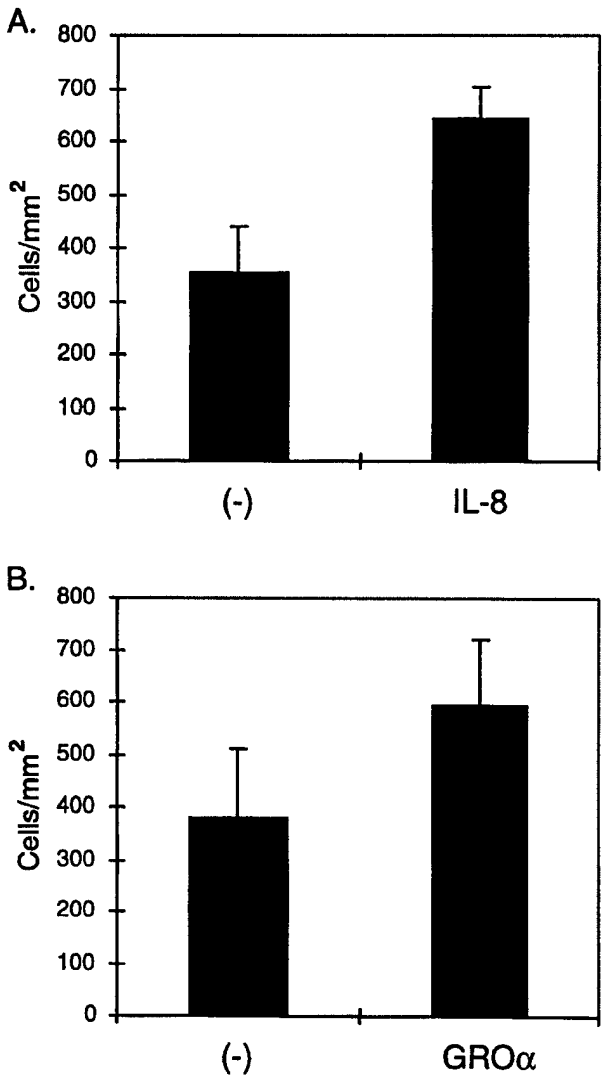

Fig. 2. IL-8 and GROα stimulate invasion through a reconstituted basement membrane. PC3 cells were added to the upper well of the invasion chamber and allowed to invade through the reconstituted basement membrane towards (**A**) 36 nM IL-8 or (**B**) 13 nM GROα. Invasion assays were for 30 hr. Cells that invaded attached to the bottom of the supporting filter, which was coated with fibronectin. Cells were stained and visually quantified. Data represent the means of triplicate determinations plus or minus the standard error of the mean.

To examine if another member of the CXC-chemokine family could also stimulate migration, we tested whether GROα could also stimulate migration. At the nanomolar concentrations used in these experi-

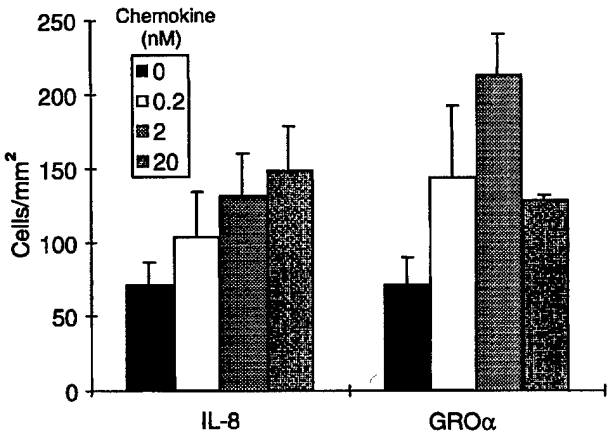

Fig. 3. IL-8 and GROα are chemotactic for PC3 cells migrating on laminin. Chemokines at the indicated concentrations were added to the lower well of a modified Boyden chamber. The wells were overlaid with filters coated on both sides with 5 μg/ml laminin. PC3 cells were added to the upper wells and incubated for 6 hr, and cells that migrated through the filter were stained and visually quantitated. Data are from representative experiments and are the means of triplicate plus or minus the standard deviation of the mean.

ments, GROα acts through CXCR2 and does not bind CXCR1 [16]. GROα also stimulated PC3 chemotaxis on laminin, indicating that CXCR2 is functional in PC3 cells (Fig. 3). At 0.2 nM, GROα PC3 cells responded with a 1.7-fold increase in migration toward the chemokine over basal migration ($P < 0.01$), with maximal response at 2 nM GROα ($P < 0.001$). The decrease in GROα stimulation of chemotaxis at 20 nM was occasionally seen at higher concentrations of both IL-8 and GROα, and is seen in other systems [32].

IL-8 and GROα Stimulate Transient Changes in Adhesion to Laminin

Changes in the adhesive phenotype can contribute to an increase in migration behavior. Therefore we looked at whether IL-8 stimulates laminin-mediated adhesion in a short-term adhesion assay. PC3 cells were pretreated for 0–30 min with 18 nM IL-8, added to laminin-coated wells, and allowed to adhere for 35 min. Suboptimal laminin concentrations were used so that when no chemokine was added, the laminin concentration supported only very low levels of cell adhesion. Preincubation with IL-8 for 15 min resulted in a 2-fold increase in adhesion ($P < 0.01$) (Fig. 4A). The increase in adhesion was transient, as the cells did not continue to respond to IL-8, returning to unstimulated levels after 30 min of IL-8 treatment.

GROα stimulation of adhesion was also observed, but was greater than that observed with IL-8 (8.9-fold

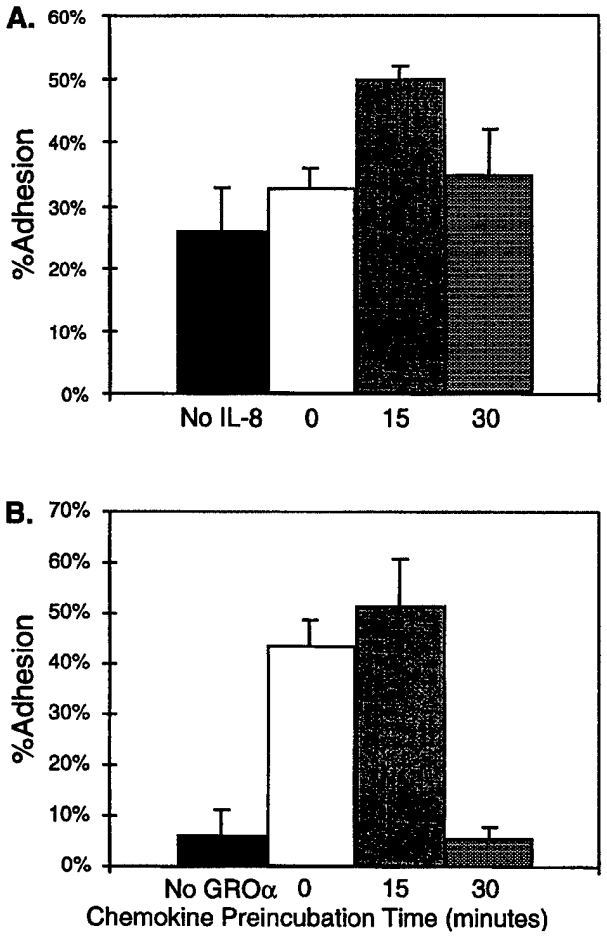

Fig. 4. IL-8 and GROα stimulate PC3 adhesion to laminin. Fluorescently labeled PC3 cells were pretreated with (**A**) 18 nM IL-8 or (**B**) 4 nM GROα for the indicated times (in min) and then added to microtiter plates coated with laminin. Cells were allowed to adhere for 30 min and then were washed, and adherent cells were quantified with a fluorescent plate-reader. Cells with no preincubation had chemokine added at the start of the assay. Adhesion of treated cells is compared to untreated PC3 cells.

vs. 2-fold, Fig. 4B). GROα increased PC3 adhesion to laminin when added at the start of the adhesion assay. Although GROα stimulated PC3 adhesion laminin more rapidly than did IL-8 (Fig. 4), this difference was not routinely observed (data not shown). In all experiments, the IL-8- or GROα-stimulated increase in adhesion was transient, as adhesion returned to prestimulation levels after 30 min. The laminin adhesion receptor levels did not change over the time course of the adhesion assay as measured by flow cytometry, and anti-α6 or anti-β1 integrin antibodies inhibited prostate cell adhesion to laminin in the presence or absence of chemokines (data not shown). These results indicate that chemokine-stimulated increases in laminin-mediated adhesion require laminin-specific inte-

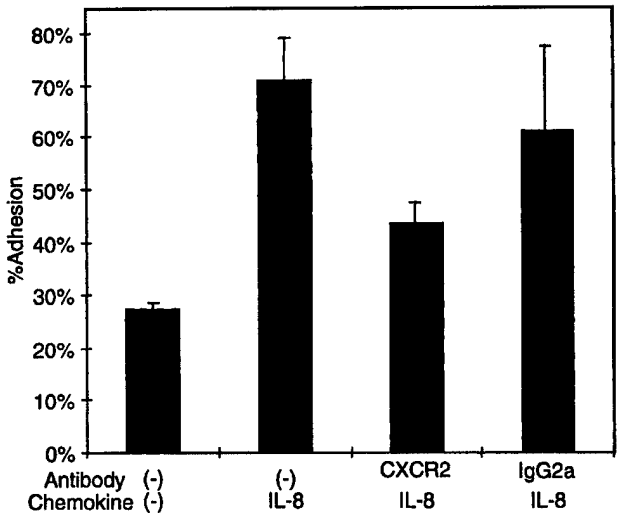

Fig. 5. Neutralizing antibodies to CXCR2 inhibit IL-8 stimulation of adhesion to laminin. PC3 cells, preincubated with or without 0.5 μg/ml anti-CXCR2 mAb or control isotype-matched antibody, were stimulated with 10 nM IL-8 for 10 min and added to adhesion wells coated with 2.5 μg/ml laminin, and then were incubated for 35 min, washed, and quantified.

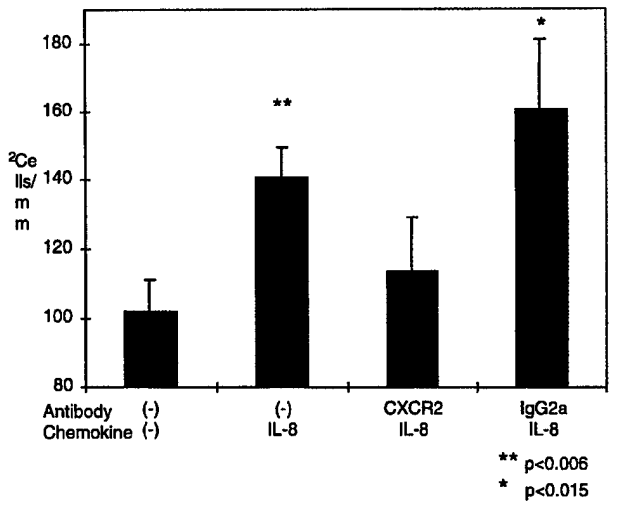

Fig. 6. Neutralizing antibodies to CXCR2 inhibit IL-8 stimulation of chemotaxis. PC3 cells, preincubated with or without 0.5 μg/ml anti-CXCR2 mAb or control isotype-matched antibody, IgG2a, were added to the chemotaxis assay in which 10 nM of IL-8 were added to the lower chamber. Migration was for 6 hr on a filter coated on both sides with 7.5 μg/ml LMN. *$P < 0.015$. **$P < 0.006$.

grins, and they suggest that chemokines stimulate functional changes in these integrins.

IL-8 Utilizes the CXCR2 Receptor to Stimulate Adhesion and Migration

IL-8 binds to both CXCR1 and CXCR2 with high affinity, and both receptors are able to signal adhesion and chemotaxis. As PC3 cells express message for CXCR2 and respond to chemokines which bind only CXCR2 at nanomolar concentrations, we used neutralizing antibodies for CXCR2 in the adhesion and chemotaxis assays to block IL-8 stimulation of adhesion and migration. The IL-8 adhesion was mediated by the CXCR2 receptor, as neutralizing antibodies to the receptor inhibited 65% of the IL-8 stimulation of adhesion ($P < 0.003$) (Fig. 5). The CXCR2 antibody also inhibited 70% of the IL-8 stimulation of migration (Fig. 6).

GROα-Stimulated Migration of Prostate Carcinoma Cells Through $\alpha_6\beta_1$ Integrin

PC3 cells use $\alpha_{6\beta1}$ integrin to adhere and migrate on laminin. The β_1 integrins are a major family of adhesion molecules used to bind to matrix molecules such as laminin. The CXC-chemokines have been shown to stimulate adhesion through the β_1 integrins on T lymphocytes [8,33]. Neutralizing antibodies to β_1 integrin and α_6 integrin subunits inhibit both basal migration and GROα-stimulated migration of PC3 cells, indicating that prostate carcinoma cells treated with GROα use $\alpha_6\beta_1$ integrin to migrate on laminin (Fig. 7). Further experiments with inhibiting antibodies to integrins did not suggest a role for α_2, α_3, α_4, α_5, or β_4 integrin subunits in chemokine-induced PC3 cell migration on laminin (Data not shown). In addition, inhibiting antibodies showed that the $\alpha_6\beta_1$ integrin was used for IL-8-stimulated migration and adhesion on laminin (data not shown). IL-8 and GROα also both stimulated migration on fibronectin at 2 and 20 nM chemokine ($P < 0.01$) (Fig. 8), possibly through effects on the fibronectin integrins $\alpha_5\beta_1$ and $\alpha_3\beta_1$, both of which are expressed by PC3 cells. This suggests that the CXC-chemokines may act on the β_1 integrins to enhance migration and invasion. As was observed for $\alpha_6\beta_1$ integrin, cell surface levels of the fibronectin integrins $\alpha_5\beta_1$ and $\alpha_3\beta_1$ did not change as a function of chemokine treatment. These results suggest that IL-8 and GROα stimulate migration of PC3 cells by modifying the function of the existing β_1 integrins rather than by upregulating expression of new adhesion receptors, as has been demonstrated in leukocytes.

DISCUSSION

Chemokines are important for stimulating cell adhesion, chemotaxis, and invasion of inflammatory cells [1]. Chemokines also promote adhesion and migration of other cell types, including breast carcinoma

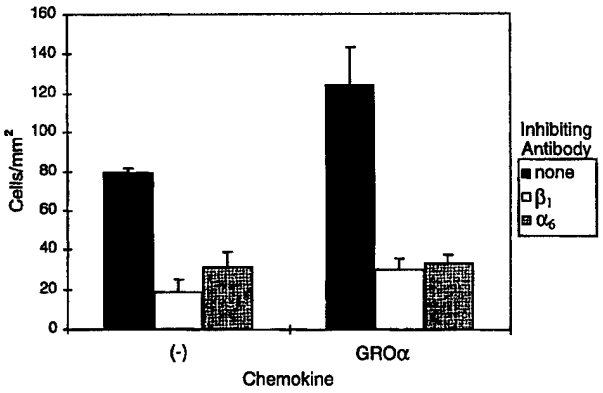

Fig. 7. Neutralizing antibodies to β_1 integrin and α_6 integrin inhibit GROα stimulation of chemotaxis. PC3 cells preincubated with or without 0.5 μg/ml anti-integrin mAbs were added to the chemotaxis assay in which no chemokine or 2.5 nM GROα were added to the lower chamber. Migration was for 6 hr on a filter coated on both sides with 7.5 μg/ml LMN.

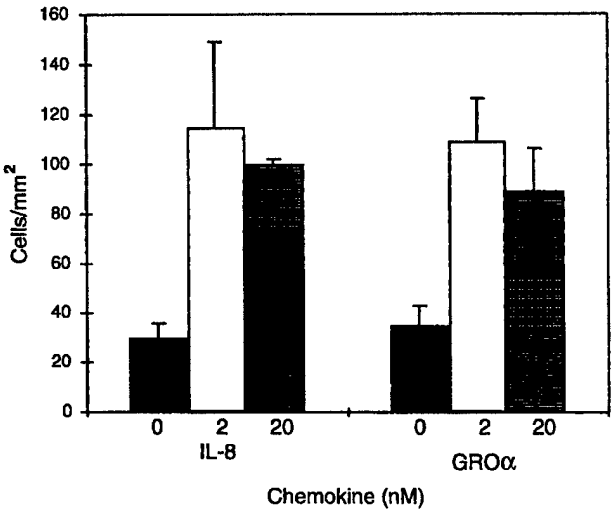

Fig. 8. IL-8 and GROα are chemotactic for PC3 cells migrating on fibronectin. PC3 cells were incubated for 6 hr in modified Boyden chambers with filters coated with 5 μg/ml fibronectin and chemokine in the lower well as the chemoattractant.

and melanoma cells, indicating a role for these chemotactic cytokines in tumor migration and invasion [9,10]. We therefore examined whether two CXC-chemokines, IL-8 and GROα, could promote prostate carcinoma-cell invasion, migration, and adhesion. CXC-chemokines, IL-8 and GROα, stimulate PC3 carcinoma-cell invasion through reconstituted basement membrane. The increase in invasion is accompanied by an IL-8- and GROα-stimulated increases in chemotaxis of cells on laminin, a major component of the basement membrane. In addition to stimulation of invasion and migration, which take place over a period of 6–24 hr, the CXC-chemokines stimulate transient increases in adhesion, which are maximal at 15 min.

Several lines of evidence demonstrate that the chemokine receptor, CXCR2, contributes to these biological effects. First, PC3 cells express mRNA for CXCR2. Next, neutralizing antibodies to CXCR2 inhibit 60% of the IL-8 stimulation of adhesion and migration. Also, CXCR2 is the only known receptor to bind GROα and stimulate migration at the concentrations used in these experiments. While CXCR2 is the predominantly active receptor in stimulating CXC-chemokine activity in PC3 cells, CXCR1 could also contribute to the IL-8 stimulation of adhesion and migration. Indeed, preliminary experiments suggest that CXCR1 contributes to IL-8-induced adhesion and migration, as anti-CXCR1 mAbs inhibit adhesion and migration when used together with CXCR2 antibodies (data not shown). Additional studies using alternate approaches to selectively isolate the two different receptors' functions are in progress to evaluate their relative contributions to chemokine-enhanced adhesion, migration, and invasion.

The mechanism by which IL-8 and GROα stimulate invasion is not known. The transient nature of the adhesion effect may be important for regulating chemotaxis and invasion, as the migration of cells involves both adhesive and deadhesive events. Transient stimulation of adhesion would allow the cell to attach at the leading edge and then migrate, rather than remain firmly attached in one place. It has been proposed that chemokines stimulate more than one signal transduction pathway, leading to the same chemokine being able to stimulate both short-term adhesion and long-term migration and invasion [33]. In addition to adhesion and migration, degradation of the matrix is also an important step in invasion. IL-8 and GROα are known to stimulate neutrophils to release preformed granules containing proteinases [34]. Whether the CXC-chemokines stimulate protease expression or activity on prostate carcinoma cells is not known. PC3 cells are invasive without the addition of chemokines, although at reduced levels, indicating that sufficient proteases are present for invasion. Additional protease release or activation could increase the invasiveness of the cells. However, as PC3 cell motility is increased by chemokine treatment, this suggests that increased migration contributes to increased invasion. The increased tumor migration and invasiveness could translate into increased metastasis of a prostate tumor in vivo when it results in cells moving away from the tumor into new tissues.

Multiple potential sources of CXC-chemokines are present in the prostate and in prostate carcinoma. Tumors are heterogeneous mixtures of cells, including immune cells that infiltrate in response to the tumor,

which could also secrete a number of chemokines. IL-8 is present in sites of inflammation, being secreted by activated monocytes, macrophages, neutrophils, endothelial cells, fibroblasts, and mitogen-activated T lymphocytes [1,35]. Macrophages, a potential source of IL-8 in prostate carcinoma, are present at higher levels in highly metastatic prostate carcinoma than in locally invasive carcinoma [36]. Although the macrophage could act in pleiotrophic ways to promote prostate tumor progression, secretion of IL-8 and the CXC-chemokines could stimulate migratory and invasive phenotypes in prostate carcinoma cells. In addition to the immune cells that produce chemokines, cells normally within the prostate also may produce CXC-chemokines [37]. In this regard, IL-8 has also been shown to be produced by stromal cells cultured from prostates, although the exact cell type(s) producing IL-8 in these cultures was not identified [37].

The CXC-chemokines could stimulate autocrine activity, as tumor cells themselves are possible sources of IL-8 and GROα [1]. IL-8 expression in tumors can be upregulated by inflammatory cytokines [38]. IL-8 expression can also be upregulated at the site of tumor implantation [39], and in areas of necrosis which are accompanied by hypoxia and ischemia [40]. Prostate adenocarcinoma cells implanted orthotopically express more IL-8 mRNA than cells placed ectopically, indicating that the microenvironment of the prostate is capable of stimulating the carcinoma cells to produce IL-8 [4]. GROα is produced by a variety of cancer cell types and is also present at sites of inflammation [1,41]. GROα has not yet been identified in the prostate or prostate carcinoma; however, it is found in seminal fluid, which is produced in part by the prostate gland [42]. The PC3 cells used in these studies expressed IL-8 protein, as determined by ELISA (personal communication from Dr. Joseph E. De Larco), and this chemokine production could contribute to the basal migration seen on laminin. Thus, IL-8 and GROα could act in an autocrine fashion to stimulate basal migration of prostate carcinoma, either in the primary tumor site or at distant sites. In addition to IL-8 and GROα, NAP-2, ENA 78, GCP-2, GROβ, and GROγ all bind to the CXCR2 receptor and are possible additional sources of chemotactic activity for prostate carcinoma in vivo [14,15]. During the course of tumor progression, as prostate tumors invade locally or metastasize to a distant site, the tumor may use CXCR2 to respond to several members of the CXC-chemokine family, depending on which chemokine family member is present at a particular site.

IL-8 and GROα stimulate prostate carcinoma chemotaxis on laminin-coated substrata through $\alpha_6\beta_1$ integrin, which has been linked to prostate tumor progression [43]. Although no clear changes in β_1 integrin expression have been found during prostate cancer progression, several reports have described relative decreases in β_4 expression in advanced prostate carcinoma compared to normal tissue [44,45]. The α_6 integrin associates with both β_4 and β_1 integrin. In more advanced prostate cancer, β_4 integrin is downregulated and the α_6 subunit preferentially associates with β_1 [46]. The relative increase in $\alpha_6\beta_1$ expression may lead to increased malignant behavior, since it was previously shown that the expression of $\alpha_6\beta_1$ integrin causes an increase in the invasiveness of the prostate cells in an in vivo experimental model [43]. IL-8 and GROα stimulation of adhesion and migration is not limited to $\alpha_6\beta_1$ integrin-specific ligands, as these chemokines also stimulate adhesion and migration on fibronectin, which binds $\alpha_5\beta_1$ and perhaps $\alpha_3\beta_1$ integrin. This suggests IL-8 and GROα may stimulate β_1 integrin-mediated events in general rather than acting through one particular α/β integrin heterodimer. As a result, chemokines may act to stimulate invasion of prostate carcinoma cells through multiple tissues and basement membranes, thus contributing to metastasis during several stages of the process.

IL-8 and GROα stimulate adhesion and migration through modulation of the activation state of β_1 integrins, rather than by changing the surface expression levels of these integrins. In other cell types, the CXC-chemokines stimulate β_1 integrin-mediated adhesion and migration on matrix components. IL-8 has been shown to stimulate T-cell [8], neutrophil [47], and sickle erythrocyte [48] migration or adhesion on fibronectin and fibrinogen. β_1 integrin receptor expression is not profoundly changed by the chemokines, and this has led to the proposal that the β_1 integrins are functionally activated by the CXC-chemokines [47]. In prostate carcinoma cells, this is supported in part by the observations that IL-8 and GROα can transiently induce high levels of PC3 cell adhesion at laminin concentrations that normally support only low levels of adhesion. Also, PC3 cells use the same integrins for chemokine-induced migration as for basal migration. Finally, IL-8 and GROα do not upregulate β_1 integrin expression in prostate carcinoma cells. Therefore it may be possible that changes in receptor affinity or avidity rather than expression account for the stimulation in prostate carcinoma cells. The $\alpha_6\beta_1$ integrin can be activated by stimulating inside-out signaling pathways [49,50], and studies are in progress to evaluate if chemokine treatment alters the relative affinity of PC3 cells for laminin. Alternatively, the chemokines could be changing integrin avidity or they may impact on signal pathways downstream of the integrin to enhance integrin-mediated matrix interactions.

The chemokines are known to stimulate signal

transduction pathways that are linked to integrin activation. IL-8 and GROα stimulate intracellular calcium release [51], PI3 kinase [52], and Rho activity [22], which are also linked to increased integrin-mediated adhesion, cytoskeletal changes, and integrin activation [22,53–55]. Inhibiting the IL-8 signal transduction pathways that have been linked to integrins inhibits the ability of the chemokine to stimulate migration and adhesion. For example, inhibiting PI3 kinase inhibits IL-8-stimulated migration [52], and inhibiting Rho inhibits IL-8-stimulated adhesion of lymphocytes transfected with IL-8 receptors [22]. Chemokines have been demonstrated to increase T lymphocyte β_1 integrin-mediated adhesion [33,56] and migration [33] to matrix molecules without changes in integrin expression, suggesting changes in integrin activity [56]. Chemokine stimulation of migration and adhesion in T lymphocytes (similar to results with prostate cells) suggests that chemokines use more than one signal transduction pathway to stimulate such a broad range of effects [33]. By using more than one signal transduction pathway in tumors, the CXC-chemokines could stimulate both more long-term (invasion, migration) and short-term (adhesion) effects.

In conclusion, the CXC-chemokines, which stimulate PC3 prostatic carcinoma cells to adhere to, migrate on, and invade the basement membrane, could be important in signaling the tumor cell to become invasive. The CXCR2 receptor, present on PC3 cells, mediates the CXC-chemokine stimulation of adhesion, migration, and invasion. As the CXCR2 receptor binds many members of the CXC-chemokine family, prostate cells could respond to a variety of CXC-chemokines, allowing the tumor to take advantage of different chemokines at different points in tumor progression. Focusing on how the receptor signals tumor cells may provide basic insights into how tumors invade surrounding tissue. In particular, it will be important to understand CXC-chemokine receptor-signaling in prostate cancer and to define the relationship of this receptor to β_1 integrin-mediated adhesion and migration. Factors that change integrin activity rather than gross changes in integrin expression may play an important role in signaling the adhesion and migration changes required for the prostate carcinoma to become invasive. Understanding pathways between the CXC-chemokines and integrin action may provide better diagnoses or may lead to an effective therapy for the management of prostate cancer patients.

ACKNOWLEDGMENTS

The authors thank Dr. Joseph E. De Larco for excellent advice during the course of these studies. This research was supported by National Cancer Institute/National Institutes of Health grant CA29995 to L.T.F. and DOD grant PC970519 to J.B.M.

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